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14. ABSTRACT

The intent of this project is to integrate whole exome genetic and global expression data to identify genes that contribute to the formation, progression and heterogeneity of NF2-associated tumors. To date we have prepared and submitted for exome sequencing at a sequencing core facility 126 samples representing paired human tumor (meningioma or schwannoma) and normal DNAs from the same individuals. We have also prepared RNA from the same tumors for transcriptome sequencing. Generation of these valuable datasets is on track for completion and subsequent individual and integrated analyses. In advance of the need to confirm the expected findings, we have generated a novel cell system using advanced genome editing technology that will permit the assessment of cellular phenotypes resulting from the genetic and gene expression changes identified.

15. SUBJECT TERMS
Neurofibromatosis 2, meningioma, schwannoma, exome, transcriptome

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Introduction

In neurofibromatosis 2 (NF2), tumor formation requires inheritance of a germline mutation in the *NF2* gene that inactivates one allelic dose of the merlin tumor suppressor, followed by somatic loss or inactivation of the remaining allele. However, it is not known whether merlin deficiency alone is sufficient for tumor formation, either in schwannomas or in meningiomas, the two major tumor types of NF2. A number of lines of evidence suggest that other genetic lesions may participate in the initiation of NF2 tumors and that other genes certainly contribute to the functional heterogeneity and progression observed in meningiomas. In the latter instance, there are many studies that have noted rearrangements of particular chromosomal regions in merlin-deficient meningiomas, but despite many years of investigation of these regions, the critical genes that participate in tumor development and heterogeneity and the biological pathways that they represent remain unknown. We postulate that there are somatic genetic events that occur in individual genes that contribute to the formation, progression and heterogeneity of tumors in NF2 and that integrated analysis of whole exome sequence data together with tumor characteristics, genomic rearrangements, RNA expression data and microRNA expression data from these tumors in comparison with normal arachnoidal cells will reveal the biological pathways that underlie the development and functional heterogeneity of NF2 meningiomas.

Body

Task 1- DNA sequence analysis of meningiomas

Our first specific aim was to perform paired whole exome sequencing of meningiomas and normal DNAs from the same individuals to identify *de novo*, somatic alterations, from point mutations and small insertion/deletions that affect protein structure to large regions of loss-of-heterozygosity (LOH). We also indicated the possibility of complementing these analyses of meningioma with similar analyses of the other NF2-associated tumor, schwannoma. The goal in Year 1 was to generate exome data from the first 30 of these meningiomas and their matching normal DNAs. We first successfully screened a large number of tumors and selected those for exome sequencing. We then generated high quality DNA from 26 meningiomas (4 sporadic and 22 NF2-associated) with matched blood DNA. We also augmented this analysis by preparing high quality DNA from 30 vestibular schwannomas (4 NF2 and 26 sporadic) all obtained through clinically-indicated surgery and their corresponding blood DNA. In addition, we prepared DNA from 8 meningiomas, 5 vestibular schwannomas, 4 nerve samples and 1 arachnoid sample from two independent NF2 autopsies. We then submitted DNA from 126 samples (63 tumor and normal DNA pairs) to the DNA Sequencing Facility for preparation of exome capture libraries, indexing and deep sequencing using Illumina HiSeq2500 DNA Analyzers. By running this larger than anticipated number of tumors in a single batch, we are avoiding the potential batch effects of splitting the analysis into two batches of ~60. Due to a particularly long sequencing queue in the core facility, these runs are not yet completed, but we expect to receive the raw exome data in the next few weeks. We are poised to move forward with its detailed analysis as soon as it arrives.

Task 2. RNA and microRNA sequence analysis of meningiomas

Our second specific aim was to perform RNA sequencing (RNA-seq) to define both gene and microRNA expression profiles relative to normal arachnoid tissue in the same set of meningiomas used for exome analysis above. The goal in Year 1 was to prepare RNA from the tumors, for sequencing in Year 2. We successfully prepared RNA from the tumors listed under Task 1 and are on track with our second aim, as we are now submitting the RNA for transcriptome sequencing.

Task 3. Identification and validation of genes contributing to tumorigenesis

This task comprises specific aims 3 and 4 and is not slated to begin until Year 3. Our third specific aim, to use integrated analysis to identify the genes/pathways that are implicated by somatic alterations as cooperating in tumor formation or progression to a higher grade tumor, will now actually begin later in Year 2 when the first batches of data from the exome and RNA sequencing are both available. Our fourth specific aim was to test whether changes associated with alterations of tumor-associated genes in Aim 3 are reproduced by their knock-down in normal and merlin-suppressed arachnoidal cells and whether these genes suffer somatic mutation in NF2 schwannomas. Our plan was to use the phenomenon of RNA interference, specifically

employing lentiviral-delivered short-hairpin RNAs (shRNA), to specifically suppress the expression of target genes. While this is a valid approach with which we have much experience, technological advances have provided an alternative, far better and more controlled route to achieving specific dosage control of expression of targeted genes. It is now possible to introduce targeted mutations into the appropriate human cell type, arachnoidal cell or Schwann cell. This advance is based upon the use by bacteria of the clustered regularly interspaced short palindromic repeats (CRISPR) system, which relies on CRISPR RNAs (crRNAs) in complex with CRISPR-associated (Cas) proteins to direct degradation of complementary sequences as a protection against viral and plasmid DNAs. Manipulation of this system has provided the capacity to perform highly efficient site-specific engineering of mammalian genomes [1-3].

As a starting point, to examine the effect of *NF2* loss in human arachnoidal cells (ACs), we generated isogenic AC lines, with and without *NF2* inactivation, using the CRISPR-Cas genome editing methodology. Dual lentiviral constructs encoding both a single guide RNA (sgRNA) and the Cas9 enzyme were generously provided by the Zhang laboratory (The Broad Institute and MIT) and have been recently reported [4]. The NF2-1 sgRNA is specific for human *NF2* (exon 8) and serves to target the Cas9 enzyme resulting in various insertions/deletions (in/dels) on the genomic level leading to a premature stop codon in the targeted *NF2* region and NF2 protein inactivation. We generated *NF2* (exon 8) mutations by transiently transfecting hTERT immortalized ACs with the lenti-CRISPR-NF2-1 plasmid employing an Amaxa Nucleofector II apparatus. Briefly, 1 µg of lenti-CRISPR-NF2-1 was transfected into 0.4 x10^6 ACs according to the manufacturer's instructions (Basic Nucleofector kit for primary mammalian cells, program U-023). Following transfection, the ACs were resuspended in culture medium and sparsely plated in order to manually pick individual colonies derived from single cells. After 14 days of culturing, each isolated colony was split into two parts and used for 1) *NF2* (exon8) genotyping by PCR and sequencing and 2) continued culturing and expansion of the clonal cell line. In total, 11 isolated clones were picked and genotyped, and we observed 5 wildtype (WT) clones, 3 compound heterozygous mutant clones, and 3 homozygous mutant clones. A subset of clones was expanded and underwent a second confirmatory genotyping as well as immunoblotting analysis (see Table 1 below). Our intent is to use these lines as the basis for comparison in Aim 4 with additional mutations of other genes identified in aims 1-3.

Cell line	clone #	genotype	*NF2* mutation	confirmed	Western result
AC (hTERT)	A3	wild type	none	yes	Merlin positive
AC (hTERT	A4	compound heterozygote	1) 967del23bp, 263fs > 274X 2) 984 insC, 269fs > 275X	yes	Merlin negative
AC (hTERT	A17	compound heterozygote	1) 975del8bp, 265fs > 274X 2) 982 insT, 268fs > 274X	yes	Merlin negative
AC (hTERT	A19	homozygote	967del35bp, across E8/I9	yes	Merlin negative

Table 1. Isogenic human arachnoidal cell (AC) clones with *NF2* (exon 8) inactivating mutations generated by CRISPR-Cas genome editing.

Key Research Accomplishments

- Preparation and submission for exome sequencing of 126 samples representing paired human tumor (meningioma or schwannoma) and normal DNAs from the same individuals
- Preparation of RNA from the same tumors for transcriptome sequencing
- Preparation of isogenic arachnoidal cells with or without inactivation of NF2, generated using CRISPR/Cas genome editing technology

Reportable Outcomes

The results are yet to be reported.

Conclusion

The goal of integrating genetic and gene expression data from NF2-associated tumors awaits the analysis of exome and RNA sequencing data, but the generation of these valuable datasets is on track for completion. In advance of the need to confirm the findings, when available, we have generated a novel cell system using advanced genome editing technology that will permit the assessment of cellular phenotypes resulting from the genetic and gene expression changes identified.

References

[1] Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., and Church, G. M. (2013) RNA-guided human genome engineering via Cas9, *Science 339*, 823-826.

[2] Musunuru, K. (2013) Genome editing of human pluripotent stem cells to generate human cellular disease models, *Dis Model Mech*. *6*:896-904.

[3] Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., and Jaenisch, R. (2013) One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering, *Cell 153*, 910-918.

[4] Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G., et al. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343, 84-87.